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Chemical and enzymic oxidation by tyrosinase of 3,4-dihydroxymandelate

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Tyrosinase usually catalyses the conversion of monophenols into *o*-diphenols and the oxidation of diphenols to the corresponding *o*-quinones. Sugumaran [(1986) Biochemistry **25**, 4489–4492] has previously proposed an unusual oxidative decarboxylation of 3,4-dihydroxymandelate catalysed by tyrosinase. Our determination of the intermediates involved in the reaction demonstrated that 3,4-dihydroxybenzaldehyde is not the first intermediate appearing in the medium during the enzymic reaction. Re-examination of this new activity of tyrosinase has demonstrated that the product of the enzyme action is the *o*-quinone, which, owing to its instability, evolves to the final product, 3,4-dihydroxybenzaldehyde, by a chemical reaction of oxidative decarboxylation.

INTRODUCTION

Tyrosinase (monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1), also known as polyphenol oxidase, is a copper-containing mono-oxygenase widely distributed in the phylogenetic scale. It is responsible for melanization in animals and browning in plants, and plays an important role in cuticle formation in insects. The pathway of melanization from L-tyrosine starts by two enzymic steps and continues by a series of chemical steps until the first stable product, dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone), is obtained. Thus tyrosinase has two catalytic activities: (*a*) cresolase or monophenolase, producing hydroxylation of L-tyrosine to L-dopa (3,4-dihydroxyphenylalanine), (*b*) catecholase or diphenolase activity, producing oxidation of L-dopa to *o*-dopaquinone [4-(2-carboxy-2-aminoethyl)-1,2-benzoquinone], which is the final enzymic product.

For the transformation of this *o*-dopaquinone, which is very unstable, there are two competitive pathways (García-Carmona *et al.*, 1982; García-Cánovas *et al.*, 1982), one yielding dopachrome and the other *p*-topaquinone [5-(2-carboxy-2-aminoethyl)-2-hydroxy-1,4-benzoquinone], the latter slowly evolving into dopachrome (Graham & Jeffs, 1977).

But, as is well known, tyrosinase has other substrates apart from L-tyrosine and L-dopa. The broad specificity of substrates shown by tyrosinase and the high reactivity of the product of the enzymic reaction (*o*-quinone) (García-Cánovas *et al.*, 1982; Jiménez *et al.*, 1984*a*,*b*; Cabanes *et al.*, 1987; García-Carmona *et al.*, 1987*a*,*b*) have led some authors to ascribe some new activities to the enzyme, such as hydroxylation of 3,4-dihydroxyphenylalanine to 5-hydroxydopa (Hansson *et al.*, 1980) and hydroxylation of 2,4-dihydroxyphenylalanine to 6-hydroxydopa (Morrison & Cohen, 1983); in both cases ascorbic acid was used as a cofactor. The first of these activities is actually the chemical transformation of *o*-dopaquinone (the enzymic product), as has previously been discussed (García-Cánovas *et al.*, 1982), and the second is merely an example of the cresolase activity of tyrosinase on a specific substrate. Apart from these activities, studies performed with cuticular enzyme (Lipke *et al.*, 1983; Sugumaran & Lipke, 1983*a*,*b*) on the mechanism of cuticular tanning and the β-cross-link proposed by Andersen (1979) have suggested another new activity for cuticular tyrosinase, in which a new oxidative decarboxylation of 3,4-dihydroxymandelic acid to 3,4-dihydroxybenzaldehyde is proposed. This new activity has been further generalized to mushroom tyrosinase (Sugumaran, 1986). In the present paper, we have re-studied the oxidative decarboxylation of 3,4-dihydroxymandelate, using mushroom tyrosinase and $NaIO_4$ as oxidants. All intermediates were characterized. Stopped-flow techniques were used to discover whether the oxidative decarboxylation is catalysed by tyrosinase or is due to the specific characteristics of the substrate used.

MATERIALS AND METHODS

Mushroom tyrosinase (monophenol mono-oxygenase, EC 1.14.18.1; 2480 units/mg), 3,4-dihydroxymandelic acid and L-ascorbic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; Fluka A.G., Buchs, Switzerland, supplied 3,4-dihydroxybenzaldehyde. $NaIO_4$ and all other chemicals used were of analytical grade. Measurements were carried out with an UV/Vis Uvikon 810 spectrophotometer interfaced on-line with an Olivetti M-24 computer. Spectra were recorded on the same instrument.

Rapid kinetic measurements were carried out with a SFM-3 Biologic stopped-flow spectrophotometer equipped with a xenon lamp and interfaced on-line with an IBM PC computer.

The results shown in the Figures were the averages of five measurements.

RESULTS AND DISCUSSION

Fig. 1 shows the spectra of $NaIO_4$ and 3,4-dihydroxymandelic acid, and also that of 3,4-dihydroxybenzaldehyde, which is obtained when 3,4-dihydroxymandelic

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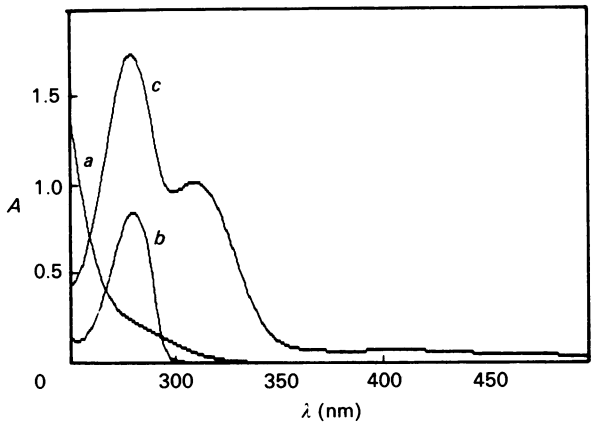


Fig. 1. (*a*) Spectrum of $NaIO_4$ (0.66 mM) in sodium acetate buffer (pH 5); (*b*) spectrum of 3,4-dihydroxymandelic acid (0.3 mM) in the same buffer; (*c*) oxidation of 3,4-dihydroxymandelic acid (0.3 mM) by $NaIO_4$ (0.133 mM) in 50 mM-acetate buffer (pH 5)

acid is oxidized by $NaIO_4$. As is well known, $NaIO_4$ is a specific oxidant which transforms *o*-diphenols to the respective *o*-quinones (Graham & Jeffs, 1977). Sugumaran (1986) studied the oxidation of 3,4-dihydroxymandelic acid catalysed by mushroom tyrosinase, and concluded that 3,4-dihydroxybenzaldehyde was the direct enzymic product. However, as pointed out above, the same product is obtained when $NaIO_4$ is used instead of tyrosinase. So, in view of these results, it is probable that the oxidative decarboxylation is a chemical property of the product obtained by using tyrosinase or $NaIO_4$ rather than a one-step catalysis by tyrosinase on 3,4-dihydroxymandelic acid.

A series of experiments with L-ascorbic acid was performed to study the activity of mushroom tyrosinase on 3,4-dihydroxymandelic acid. The non-enzymic oxidation of the ascorbate can be coupled to the enzymic formation of *o*-quinone (Pomerantz, 1963) in accordance with the following reactions:

$$o\text{-Diphenol} \xrightarrow[\text{Tyrosinase}]{O_2} o\text{-quinone}$$

$$o\text{-Quinone} + \text{ascorbate} \rightarrow o\text{-diphenol} + \text{dehydroascorbate}$$

Ascorbate oxidation is observed by a decrease in the A_{265}; it is not inhibitory and is not oxidized by the enzyme. Fig. 2 shows the data recordings obtained when mushroom tyrosinase acts on 3,4-dihydromandelic acid in the presence of variable amounts of ascorbate at pH 5. In Fig. 2(*a*), the wavelength utilized was 265 nm to follow the ascorbate oxidation, whereas in Fig. 2(*b*) the recordings were performed at 312 nm to observe the accumulation curve of 3,4-dihydroxybenzaldehyde, which showed a lag period.

By comparing both recordings (Figs. 2*a* and 2*b*), at 265 nm and 312 nm, it can be noted that in each case the time needed for L-ascorbic acid present in the reaction medium to be consumed (Fig. 2*a*) coincided with the lag period in the accumulation curve of 3,4-dihydroxybenzaldehyde (Fig. 2*b*). This fact supposes that in effect the enzymic product was the *o*-quinone; this explains the non-appearance of 3,4-dihydroxybenzaldehyde in the

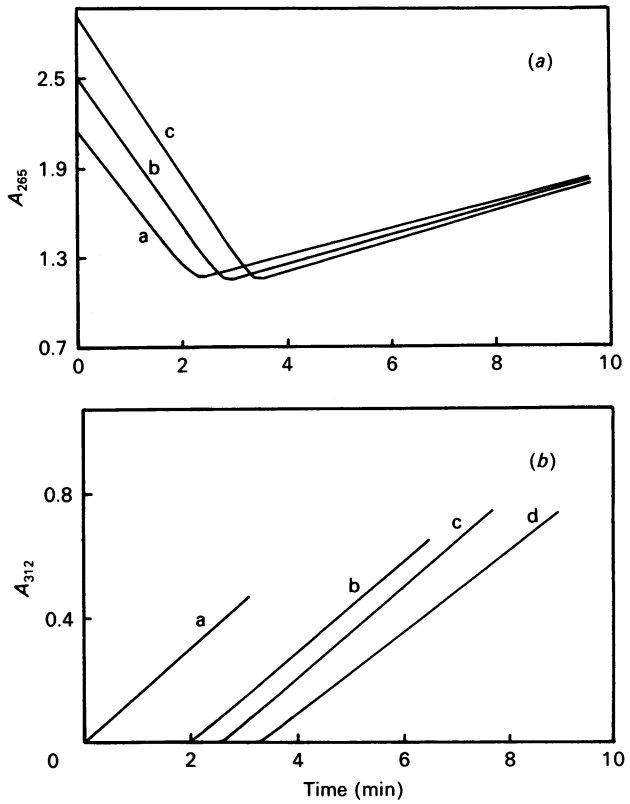


Fig. 2. Oxidation of 3,4-dihydroxymandelic acid by mushroom tyrosinase in the presence of L-ascorbic acid at pH 5

Reaction mixtures contained 1 mM-3,4-dihydroxymandelic acid, 9.5 μg of mushroom tyrosinase, 50 mM-acetate buffer, pH 5.0, and variable amounts of L-ascorbic acid (a, 83.3 μM; b, 116.6 μM; c, 150 μM). (*a*) Ascorbate oxidation was monitored at 265 nm. The A_{265} increase from a minimum at about 3 min to 10 min is due to the appearance of 3,4-dihydroxybenzaldehyde in the reaction medium; see Fig. 1(*c*). (*b*) Accumulation curve of 3,4-dihydroxybenzaldehyde, monitored at 312 nm: a, without L-ascorbic acid; b, with 83.3 μM-, c, 116.6 μM-, and d, 150 μM-ascorbic acid.

presence of L-ascorbic acid, since the *o*-quinone reacts with the L-ascorbic acid so that it cannot evolve to 3,4-dihydroxybenzaldehyde by the chemical step of oxidative decarboxylation. The same experiment was performed at pH 3.5, and similar results were obtained (results not shown).

The results obtained suggest that the enzymic product is the *o*-quinone, and only when this *o*-quinone has been obtained (the enzymic product) does it suffer a series of chemical reactions that lead to the final product, 3,4-dihydroxybenzaldehyde, owing to its instability (Scheme 1). Direct evidence to demonstrate this argument is the determination of intermediates during the first moments of the reaction. Because the period of time involved is very short, we had to use stopped-flow techniques for this study of rapid kinetics.

The *o*-quinone product was measured at its $\lambda_{max.}$, 400 nm, and the accumulation curve of 3,4-dihydroxybenzaldehyde was obtained at 330 nm (this wavelength did not correspond to the $\lambda_{max.}$ of this product). Fig. 3 shows the recordings obtained at both wavelengths under the same experimental conditions. The accumu-

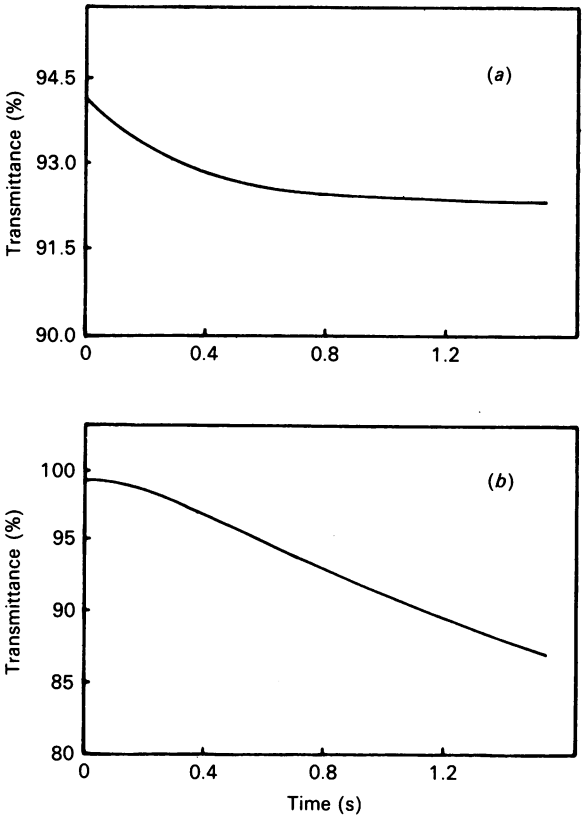


Fig. 3. Changes in transmittance (*a*, at 400 nm; *b*, at 330 nm) after the oxidation of 3,4-dihydroxymandelic acid by mushroom tyrosinase

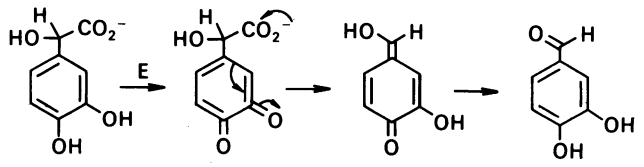
The reaction mixture was mixed in a stopped-flow apparatus with an equal volume of a medium containing 10 mM-3,4-dihydroxymandelic acid in 50 mM-acetate buffer, pH 3.5, and mushroom tyrosinase (0.15 mg/ml) in the same buffer.

lation curve of *o*-quinone presents a burst (curve *a*) corresponding to a direct product of the reaction, whereas the 3,4-dihydroxybenzaldehyde curve (curve *b*) shows a lag period, which indicates that this product is formed from the first one.

By using the same experimental conditions, the accumulation curve of *o*-quinone was followed for 90 s. The *o*-quinone reached a steady-state amount during the enzymic reaction, and when the enzymic reaction had finished this *o*-quinone developed to the final product and disappeared from the reaction mixture (results not shown).

However, when discontinuous measurements were carried out, by Sugumaran (1986), in which the enzymic reaction was stopped (h.p.l.c. studies), only the final product, 3,4-dihydroxybenzaldehyde, and no other compound could be detected in the reaction mixture.

The possibility that tyrosinase catalyses a novel oxidative decarboxylation when 3,4-dihydroxymandelic acid is used as substrate has been totally ruled out on the basis of the above studies. Firstly, the same final product, 3,4-dihydroxybenzaldehyde, was obtained when $NaIO_4$ was utilized as oxidant instead of tyrosinase. Secondly, the presence of L-ascorbic acid in the reaction medium during the enzymic reaction prevents the appearance of



Scheme 1. Proposed mechanism for the oxidative decarboxylation of 3,4-dihydroxymandelic acid catalysed by mushroom tyrosinase (E)

the final product, this being demonstrated by the fact that the consumption of the L-ascorbic acid present in the medium leads to the appearance of this final product. Thirdly, the enzymic product that undergoes oxidative decarboxylation (*o*-quinone) appears in the medium, showing a burst. Fourthly, the transformation of 3,4-dihydroxymandelic acid into 3,4-dihydroxybenzaldehyde takes place with an appreciable lag period.

Further, the scheme proposed to explain the probable mechanism for the oxidative decarboxylation of 3,4-dihydroxymandelic acid catalysed by mushroom tyrosinase contrasts strongly with that proposed by Sugumaran (1986). For this compound the carboxy group, pK approx. 3.4, is shown fully ionized (actually 97.5% at pH 5.0). The formation of its quinone provides the electron-withdrawing effect which facilitates decarboxylation. The phenoxide ion (pK approx. 9.0) formed would protonate very rapidly at pH 5.0, as shown in Scheme 1. In contrast Sugumaran's (1986) scheme is not likely. It involves the un-ionized acid as substrate, although it would be over 99% ionized at pH 6.0, and abstracts a hydride ion from the *p*-hydroxy group. The latter is improbable.

Therefore, we concluded that tyrosinase catalyses the oxidation of 3,4-dihydroxymandelic acid to its corresponding *o*-quinone, which, owing to its instability, can undergo oxidative decarboxylation and lead to the 3,4-dihydroxybenzaldehyde. Because the *o*-quinonic product is very unstable and the chemical reaction of decarboxylation is very fast, 3,4-dihydroxybenzaldehyde is apparently the first detectable product.

From all the above it can be concluded that, although tyrosinase shows different specificity toward its substrates (mono- or *o*-di-phenols), its action leads always to the quinonic product, which is very unstable and can follow different chemical reactions.

According to the development of the *o*-quinonic product, it is possible to divide substrates of tyrosinase into two groups: (*a*) those which produce *o*-quinones enzymically, and which suffer an unimolecular reaction, as for 3,4-dihydroxymandelic acid and the other substrates described in the literature (García-Carmona *et al.*, 1982, 1987*a*; Jiménez *et al.*, 1984*a*,*b*); (*b*) those whose *o*-quinones can suffer second-order reactions (Cabanes *et al.*, 1987; García-Carmona *et al.*, 1987*b*). In this way tyrosinase can appear to show a new activity that is really only due to both the broad specificity of the substrate and the great instability of the enzymic product (*o*-quinone), which can undergo very different chemical reactions.

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